The Role of the Cell Surface in Neuronal Pathfinding

A diverse array of extracellular molecules guides pathway selection by growth cones

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Perhaps the organ of the human body that evokes the most astonishment, and yet is the least understood, is our brain. From the coordination of simple movements, to consciousness and thought, the function of the nervous system relies on the establishment of trillions of precise connections among the many neurons that constitute it. Building an organ of such complexity obviously cannot be left to chance. As the nervous system develops, neurons must extend their long extensions, called axons, through the embryonic environment; the leading tips of these axons, called growth cones, are used by the neurons to find their target cells and establish synaptic contacts with them.

This process can be divided into three phases with distinct properties: pathway selection, target selection, and address selection (Figure 1). First, the growth cones extend along specific pathways, often traversing long distances. Second, they reach the target region and make their initial contacts with cells. Third, the growth cones select their address, the proper synaptic partner within the target region. Remarkably, both pathway and target selection are stereotyped and mostly error free, and they are independent of the generation of action potentials by the neurons involved. In contrast, address selection involves the refinement of initially promiscuous connections through activity-dependent mechanisms (Goodman and Shatz 1993). The mechanisms underlying the first stage, pathway selection, and specifically the role of the cell surface, are the subjects of this article.

Axonal pathfinding is accomplished by the specific interaction of receptors on the surface of growth cones with molecular cues in the embryonic environment

Pathway selection can be distinguished from target selection by its independence from the final target. Most axons start growing even before the target has developed, using intermediate cues, often cells referred to as guideposts, along the way. The work on simple and accessible nervous systems of invertebrates has suggested a general strategy by which these animals build neural networks (Bate 1976, Bentley and O'Connor 1992, Goodman et al. 1984): First, pioneer neurons lay down a simple scaffold of axons using guidepost cells, and then the later developing neurons in turn use these pioneer axons as pathways, following them in close apposition (axon fasciculation). As the number of follower growth cones increases, the narrow trails established by the pioneers soon become multilane highways of great complexity. Work in vertebrate embryos (Easter et al. 1994, Kuwada 1986) suggests that the same strategy is used in the development of more complex nervous systems as well.

Ever since the first observations of neuronal growth cones by the neurohistologist Ramón y Cajal in the late nineteenth century, they have been considered microsensors of their environment. A variety of environmental cues, including electrical, physical, and chemical cues, have been invoked to describe guidance of growth cones, but recent work suggests that chemical cues play the most important role. Physical elements, such as tissue barriers or channels, are not as crucial for guidance as initially thought because growth cones are persistent and invasive. They can advance through difficult obstacles by secreting enzymes that dissolve or degrade the matrix elements (Seeds et al. 1992).

Today, the prevailing paradigm is that axonal pathfinding is accomplished by the specific interaction of receptors on the surface of growth cones with molecular cues in the embryonic environment. There is a

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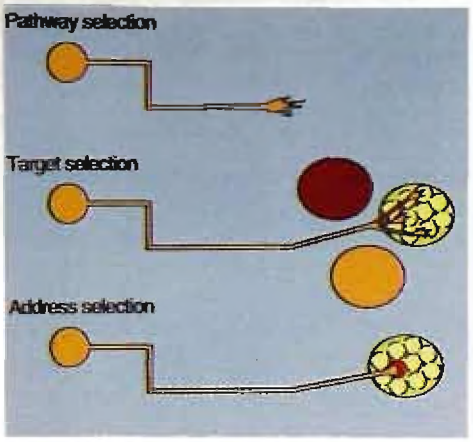

Figure 1. Three steps are necessary for the establishment of the appropriate connections in the nervous system. Neurons extend axons tipped by growth cones that must select the correct pathway to follow among the many possible directions of growth. After a sometimes complicated journey that brings it into contact with many cells, the growth cone selects a target. Within the target, the axons often form branches that initially contact many cells. Finally, the growth cone must select its final address: a small number of cells, or even a single cell, with which it establishes a functional connection—the synapse. During address selection, inappropriate synapses are eliminated by an activity-dependent process.

molecular code of information composed of cues in the environment and corresponding receptors on neuronal membranes. Growth cones must decipher the heterogeneous chemical information that they encounter in the environment to make specific pathway choices. This information is encoded by positional cues—molecules anchored to cell membranes or to the extracellular matrix (a meshlike structure that fills in the intercellular space) or soluble factors released from a guidepost or target cell. The guidance cue is detected at the cell surface by a specific receptor, which then triggers changes inside the cell that lead to a pathway choice (Doherty and Walsh 1994, Kater and Rehder 1995).

Several experimental approaches have been used to analyze the role of the cell surface in axon guidance. Analyses at both the cellular and molecular levels are now converging to contribute to understanding of axonal pathfinding.

What can we learn by observing growth cones?

Pathfinding is accomplished by the growth cone. This highly specialized structure is involved both in the elongation of the axon by assembly of new plasma membrane and cytoskeleton (the intracellular protein network that shapes the cell) and in exploration of the embryonic environment to determine the direction of growth. Direct observation of growth cones in both fixed and living preparations suggests that they are continually sniffing out guidance cues in the surrounding environment (Stirling and Dunlop 1995).

Growth cones can respond differently to different guidance cues. They can grow toward the cue or, surprisingly, they can retract or collapse to avoid the initial contact and then redirect their growth away from the cue. The phenomenon of growth cone collapse (Figure 2a) was initially described by Kapfhammer and Raper (1987), who observed growth cones in culture. Since then, many other examples have been found, both in tissue culture experiments and in living embryos (Goodman and Shatz 1993, Luo and Raper 1994, Patterson 1988). More important, these findings highlight the idea that repulsion can be as important a guidance mechanism as attraction.

When a growth cone explores its environment, it extends many long fingerlike protrusions, called filopodia, that sense external cues. The contact of a single filopodium with a guidepost cell is enough to steer a growth cone by causing it to modify its cytoskeleton (O'Connor et al. 1990). Likewise, the contact of a single filopodium with fragments of cell membranes from target cells (Müller et al. 1990) or with beads coated with a single guidance molecule (Fan and Raper 1995) can cause the growth cones of cultured vertebrate neurons to change direction, either toward or away from the cue (Figure 2b). These experiments suggest that the mechanisms mediating growth cone guidance are exquisitely sensitive: The contact of a tiny portion of the neuron's surface can generate a response to a given guidance cue.

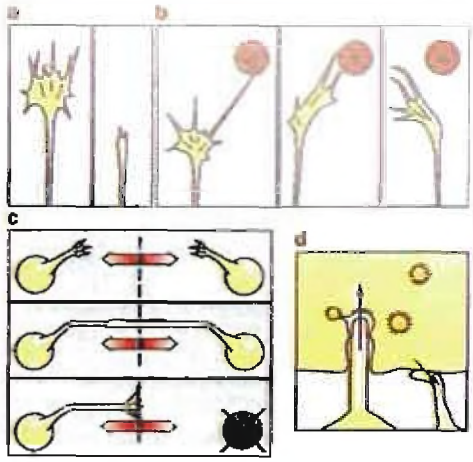

Figure 2. Cellular mechanisms underlying growth cone pathfinding. (a) Schematic illustration of the process of collapse observed in cultured neurons when growth cones receive an inhibitory cue from the environment. Growth cones extending in culture over a permissive substratum normally have a flat and broad shape with multiple filopodia exploring all directions (left). This expanded growth cone retracts its filopodia and becomes a threadlike structure (right) when a soluble inhibitory molecule, the collapsing factor, is added to the culture medium. (b) Attraction or repulsion can be accomplished when the growth cone surface contacts a localized guidance cue. The contact of a single filopodium (left) with a source of guidance cue (red sphere) is sufficient to reorient axonal growth toward (middle) or away (right) from the cue. (c) Growth cone–growth cone surface contact is necessary for insect commissural neurons to follow their pathway. The axons of neurons that cross to the other side of the embryo are directed toward the midline (upper), probably by a chemoattractant that forms a gradient (red arrows). They then contact each other and fasciculate together to grow away from the midline and down the chemoattractant gradient (middle). Contact between the two axonal surfaces is an essential requirement: When one of the neurons is killed, its partner cannot grow past the midline (lower). (d) A dialogue can be established between two growth cones. Once a filopodium has contacted another cell, information can flow in two putative directions: from the cell to the filopodium (green arrow) through cell surface receptors, and from the filopodium to the cell (blue arrow) by inducing the formation of coated pits and vesicles.

Intracellular signaling molecules act between the receptors of the cue at the surface of a filopodium and

the changes in the cytoskeleton necessary to move the growth cone; these molecules serve as an amplifying mechanism (Doherty and Walsh 1994, Kater and Rehder 1995). In particular, the level of intracellular calcium is recognized as a key second messenger that modulates growth cone motility. The analysis of cytoplasmic calcium levels in filopodia surgically isolated from the parent growth cone confirms that filopodia are fundamental elements in growth cone guidance, acting as antenna-like sensors of the environment (Kater and Rehder 1995).

Another interesting example of the requirement for growth cone contact with guidance cues is the interaction of neurons at the midline of insect embryos (Figure 2c). The bundles of axons that connect the two bilateral halves of the nervous system are called commissural tracts. One neuron on each side pioneers each commissural tract by extending an axon toward the midline, where the growth cones of the two neurons contact; both then proceed to grow in close apposition until they reach the opposite neuronal cell body. Myers and Bastiani (1993) have observed that contact of the growth cones and subsequent fasciculation of the axons are necessary for the axons to grow past the midline: If one of the cells is killed, its contralateral partner stops at the midline. This observation suggests the following model: Guidance toward the midline is caused by a gradient of a chemoattractant, a soluble molecule produced by the midline cells (Goodman 1994); the growth cones extend up this gradient, but once they reach the midline, contact with the contralateral growth cone and the strong fasciculation of both axons are necessary to overcome the midline signal and allow the axons to grow down the gradient. In this guidance system, cell surface interactions are dominant to the soluble chemoattractant gradient.

A general property of growth cones is that they need to adhere to a solid substrate to advance, and they adhere better to some substrates than to others. Consequently, the idea of using differential adhesion for growth cone guidance is certainly an attractive hypothesis. The easiest way for the growth cone to select among pathways would be to follow the more adhesive trail. However, growth cone preferences do not always correlate with the degree of adhesiveness (Luo and Raper 1994), and other specific molecular interactions may be superimposed on differential adhesion mechanisms.

In these examples we have described the growth cone surface as only a recipient of information. However, it can also converse with other cells. This sort of dialogue was seen by Bastiani and Goodman (1984) when examining the growth cones of two pioneer neurons in the grasshopper embryo with the electron microscope. Many filopodia from one growth cone insert deep within the other and induce the formation of coated pits and vesicles there (Figure 2d). This type of interaction is specific for particular pairs of cells and suggests a transfer of molecular information from one growth cone to another that might be important for their subsequent behavior.

Searching for the molecules that code for axon guidance

Cellular studies reveal the varied behaviors of growth cones during their navigation, but a description of these behaviors at the level of the molecular interactions occurring at the growth cone surface is needed to understand pathfinding. Multiple approaches have been taken to find molecules involved in axon guidance. One strategy is to generate mutant animals that have defects in axonal pathfinding. This approach can reveal cell surface, extracellular, or intracellular proteins involved in the signaling pathways. Subsequent characterization of the molecules is needed to interpret the defect caused by the mutation. Three organisms are most commonly used to identify pathfinding mutants: two invertebrates, the fruit fly *Drosophila melanogaster* and the nematode *Caenorhabditis elegans*, and one vertebrate, the zebra fish *Danio rerio*.

Another general strategy is to generate monoclonal antibodies against neuronal membrane proteins and look for expression patterns that are suggestive of a pathfinding role. The search is based on the assumption that relevant molecules exhibit a heterogeneous spatial and/or temporal distribution that enables them to provide or receive guidance information. Many organisms, both vertebrate and invertebrate, have been used for this strategy. A classic example is the embryo of the grasshopper *Schistocerca americana* (Sánchez et al. 1995b). The knowledge of the development of the grasshopper nervous system at the level of single identifiable cells has been important for the interpretation of the different expression patterns detected with the monoclonal antibodies.

The required spatial and temporal heterogeneity of guidance molecules poses a question: How many molecules are needed to provide the necessary specificity for so many different pathways? There has been considerable debate about the expected diversity of guidance molecules, ranging from the point of view that a few molecules will suffice, provided that they are precisely regulated in space and time, to the opinion that the high degree of precision requires many molecules. That controversy is fading as many molecules with different degrees of spatial or temporal restriction are being found. Molecules have been described whose expression ranges from broad patterns (e.g., on the surface of all neurons but not on non-neuronal cells) to restriction to a small subset of neurons. Moreover, some candidate molecules are regionally localized to the surface of just the axons and not the cell bodies; others are present only on small segments of the axonal surface. In the most extreme case, candidate molecules are found only on the growth cone surface.

For example, the search in grasshopper embryos using monoclonal antibodies has resulted in the identification of several molecules expressed on the surface of distinct subsets of axon bundles in the central nervous system. A map comparing the distribution of four molecules in a ganglion of the grasshopper embryo is

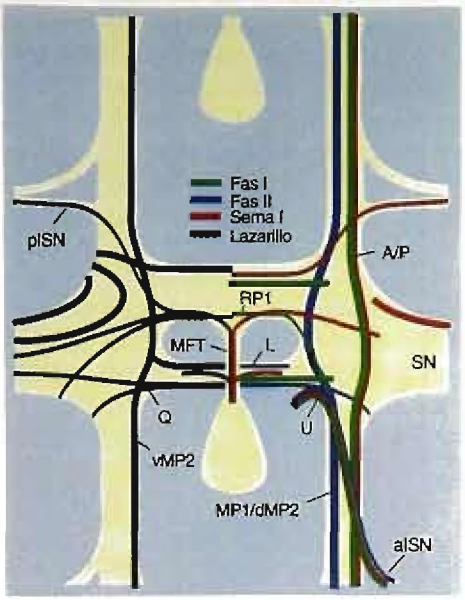

Figure 3. Diagram showing the subsets of axon fascicles that express different surface molecules in the central nervous system of the grasshopper embryo. Lazarillo is shown on the left, and fasciclin I, fasciclin II, and semaphorin I on the right. Some axon bundles, such as vMP2, possess only one of these molecules, whereas others, such as A/P, share more than one. The combination of surface molecules expressed in each bundle determines its identity and can be used as guidance information by other axons. MFT: median fiber tract; pISN: posterior branch of intersegmental nerve; aISN: anterior branch of intersegmental nerve (or U fascicle); SN: segmental nerve. A/P, MP1/dMP2, vMP2, Q, L and RP1 are fascicles named after the neurons that pioneer them.

shown in Figure 3. In each repeated segment of the embryo, a ganglion is formed near the surface of the ventral midline. Axons extending from neurons form a scaffold that is organized into longitudinal fascicles connecting successive ganglia and two commissures per segment, which connect the sides of each ganglion. In addition, an axon bundle forms at the midline, and nerves exiting from the scaffold connect the central nervous system with the periphery. Each of the cell surface molecules is expressed on axons comprising particular fascicles, and many fascicles express more than one surface molecule. These data support the hypothesis that a particular combination of molecules provides the unique specification of each axon bundle. Because these molecules are located on cell membranes, they could be the receptors for guidance cues that specify these pathways, or they could be cues for the later arriving follower axons that use this scaffold as a highway to reach their targets.

In the following sections we describe cell surface molecules that are good candidates to be part of the guidance code. Most of the candidate molecules for growth cone navigation belong to one of several families of proteins, each with different properties that can be used for guidance. We do not review all the molecules that have been found but present representative examples that illustrate the properties of the molecular code for axon guidance.

The immunoglobulin superfamily provides a wide range of different molecules for pathfinding. The immunoglobulin (Ig) superfamily consists of several proteins that contain domains resembling those of antibody molecules. It is in turn subdivided into families of more closely related proteins. The neural cell adhesion molecule (N-CAM), one of the first described and best characterized cell–cell adhesion molecules, is a membrane protein whose extracellular portion contains five repeated Ig domains. N-CAM can bind to itself, a property termed *homophilic adhesion*. This adhesion plays an important role during development in localizing the different cell types in their respective sites within tissues and organs. Because of its wide distribution in non-neuronal as well as in neuronal tissues, neurobiologists thought for years that N-CAM was just a passive adhesive substrate permissive for axonal growth or for cell migration. Superimposed on this permissive environment, other, more specific, cell surface molecules would confer directional cues.

However, N-CAM has interesting characteristics that provide a level of regulation suitable for use in axon guidance as well. It can exist in different forms depending on the amount of carbohydrate attached to it. Highly glycosylated N-CAM can contain up to 30% by weight of sialic acid, a molecule that carries a negative charge, whereas the less glycosylated forms have only 10% sialic acid. The charged sialic acid chains on N-CAM enormously reduce its adhesiveness and that of the cells that express it. It is therefore possible to influence cell adhesion by controlling the amount of the sialic acid with a fine spatial or temporal regulation.

One case in which N-CAM has been found to participate in axon guidance is in the motoneurons that innervate the limb muscles in the chick embryo (Tang et al. 1994). In these motoneurons, the sialic acid content of N-CAM is regulated both spatially and temporally. When the motoneuron axons exit the spinal cord, they are intermingled in several nerves, but at the base of the limb bud they diverge to form a plexus. As they exit the plexus the axons are reorganized and directed to the different limb muscles along a stereotyped set of pathways. Although N-CAM is always present on the surface of these axons, the sialic acid–enriched N-CAM first appears when they enter the plexus, that is, when they separate from the partners with which they were traveling and refasciculate with new partners. If sialic acid is removed from N-CAM, the motor axons make pathway errors. Tang et al. (1994) have proposed that the sialic acid modification of N-CAM is an important and active modulator that allows or prevents the interaction of other guidance molecules that could specify each one of the pathways to the appropriate target muscle.

An additional way of generating different forms of N-CAM is by controlling its RNA processing. RNA precursors are processed by splicing and joining fragments (called exons), each coding for a portion of the protein. N-CAM RNA precursor is differentially processed in some regions of the rat brain during development, producing an increasing number of mRNA molecules with a particular exon that introduces a sequence of ten amino acids into the protein. This change in N-CAM correlates with a decrease in outgrowth of neuronal processes in these brain regions. Experiments in neuronal cultures have demonstrated that the presence of the extra amino acids in N-CAM inhibits its ability to promote axonal growth (Doherty

et al. 1992).

Recently, N-CAM and other adhesion molecules have been shown to be involved in axonal extension. N-CAM may interact directly with a growth factor receptor to promote axonal growth (O'Brien 1995). These general adhesion molecules are serving more specific functions in axon growth and guidance than was previously thought. Given the broad distribution of N-CAM, one could imagine that its absence in an animal would result in severe defects. Surprisingly, mutants that lack N-CAM show defects in only a few specific cell migration processes. The effects of mutations in this and other general adhesion molecules are more subtle than expected (Müller and Kypta 1995).

Another member of the Ig superfamily is the neuron–glia cell adhesion molecule (Ng-CAM), a transmembrane protein with six extracellular Ig domains. Ng-CAM is expressed primarily on neurons and glial cells, a pattern more restricted than that of N-CAM (Thiery et al. 1985). In many cases, glial cells constitute the substrate for the migration of neurons or their growth cones. Ng-CAM binds to itself to hold axons together, but interestingly, it can also bind to a different molecule to allow axons to grow on the surface of glial cells (Grumet and Edelman 1988).

A protein related to Ng-CAM is called L1. To test L1 function, Drazba and Lemmon (1990) added antibodies against it to cultured neurons growing over a layer of glial cells. Blocking L1 in this manner produced both a decrease in axonal growth on the glial surface and the defasciculation of axon bundles. This result suggests a role for L1 in fasciculation of axons by homophilic adhesion and also in the adhesion of neurons and their axons to the surface of glia. Like N-CAM, L1 also seems to interact directly with growth factor receptors to stimulate axonal growth (O'Brien 1995). In spite of these shared functions with Ng-CAM and N-CAM, L1 possesses a higher level of spatial restriction—it is found only on portions of axons. Its expression is of particular interest in the case of commissural neurons of the vertebrate spinal cord, which are a paired neuronal group situated dorsally that send axons toward the ventral midline (Dodd et al. 1988). During this trajectory they express TAG-1 (another axonal surface protein that is an Ig superfamily member) and travel without forming bundles. After crossing the midline, the commissural axons turn off TAG-1 expression and begin expressing L1. Interestingly, at this time these axons change direction and begin to navigate in bundles. The differential expression of these two molecules correlates with the changes in direction and axonal fasciculation, supporting the hypothesis that TAG-1 and L1 are involved in axon guidance.

Although all of the Ig superfamily proteins described so far were first found in vertebrates, they are also present and highly conserved in invertebrates. For example, fasciclin II, an insect axonal glycoprotein (Bastiani et al. 1987), is similar in both amino acid sequence and adhesive properties to N-CAM. Its restriction to the surface of a subset of axon pathways in the nervous system (Figure 3) suggests that it plays a role in axon guidance.

Genetic manipulations in the fruit fly *D. melanogaster* have recently tested this hypothesis (Lin and Goodman 1994, Lin et al. 1994). Genetic analyses can be performed by preventing the expression of a protein (loss-of-function mutation), or alternatively, by increasing the expression or producing the protein in cells that normally do not express it (gain-of-function mutation). A loss-of-function mutation of fasciclin II produces defasciculation in the central nervous system, whereas an excess of this protein on the same axons produces anomalous joining of bundles that normally run separately (Figure 4b). Both results suggest an axon–axon adhesion role for fasciclin II. Increasing the levels of fasciclin II on motoneuron axons prevents normal defasciculation and, as a consequence, the axons are misrouted because they are constrained to keep growing along a bundle that they normally leave. These results are strikingly similar to those resulting from the perturbation of fasciclin II's relative, N-CAM, in the chicken motoneuron plexus.

Axon–axon adhesion is important for follower axons to take the appropriate routes along an established network of fascicles, but there are still many questions left unanswered. Simple adhesion cannot explain, for example, why axons extend along a pathway in one direction and not the other, or why they change their pathway preferences over time.

The function of another insect protein belonging to the Ig superfamily, fasciclin III (Patel et al. 1987), has also been recently analyzed using the same genetic approach (Chiba et al. 1995). This protein is expressed on the surface of a subset of neurons in the central and peripheral nervous system of the fruit fly, as well as on a few muscles of the body wall, some of which are innervated by motoneurons that express fasciclin III. Preventing the production of fasciclin III in a loss-of-function mutant does not have much effect on the wiring of the nervous system. However, if muscle cells that do not ordinarily express this protein are forced to produce it (called ectopic expression), a particular motoneuron innervates these muscles inappropriately. These experiments suggest that fasciclin III plays a role in the recognition of target cells by the growing axon, one of the last steps in the establishment of connections during nervous system development, but they also reveal that fasciclin III is not the sole determinant of motoneuron–muscle recognition and that other guidance molecules are involved in coding this specific recognition process.

Two other interesting members of the Ig superfamily are the unc-5 protein from the nematode *C. elegans* and the vertebrate molecule called F3/F11. Although these are different molecules expressed in different organisms, they share several characteristics. They are receptors for extracellular molecules with bifunctional properties that can elicit attraction or repulsion, depending on the receptor at the surface of the growth cone (see box on page 350). In particular, both of these Ig proteins are neuronal surface receptors that mediate repulsion; however, it is not yet clear whether repulsion on

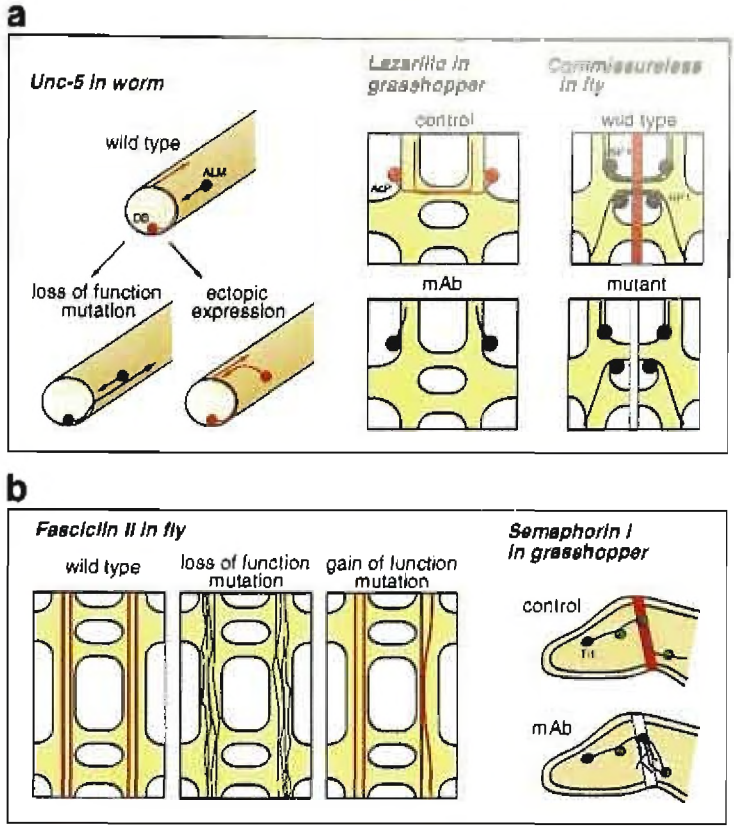

Figure 4. Experimental evidence for the involvement of some cell surface proteins in axon pathfinding. (a) Cell surface molecules that provide guidance information at the midline in different organisms. Unc-5, a protein of the Ig superfamily, is normally expressed in the DB but not the ALM neurons of the nematode *Caenorhabditis elegans*. The DB neurons normally send their axons away from the ventral midline. In a loss-of-function *unc-5* mutant, dorsal growth is impaired and the DB axon instead extends in a longitudinal direction that normally would be taken only after it reaches the dorsal midline. Conversely, inducing the expression of unc-5 in the ALM neurons causes them to take a dorsally directed pathway that they never take in normal embryos. Genetic analysis suggests that unc-5 is the receptor for a guidance signal produced at the ventral midline that mediates repulsion of the DB growth cones. Lazarillo is a surface molecule of the lipocalin family expressed in the AcP grasshopper neurons, which cross the ventral midline of the embryo. Perturbation with a monoclonal antibody causes the AcP growth cones to fail crossing the midline, and instead they follow the longitudinal pathway that they normally take at the other side of the embryo. This suggests that lazarillo is a cell surface receptor that mediates growth cone attraction toward the ventral midline. Another cell surface molecule involved in guidance toward the midline is commissureless, which is expressed in the non-neuronal cells at the midline of the fruit fly embryo. When the *commissureless* gene is mutated, the commissural neurons SP1 and RP1 do not cross the midline. (b) Cell surface molecules involved in the formation of normal axon fascicles in fruit fly and grasshopper. The function of fruit fly fasciclin II in axon fasciculation has been examined using a genetic approach. Fasciclin II, a protein of the Ig superfamily, is normally expressed on the surface of two longitudinal pathways. When no fasciclin II is present, the bundles become disorganized, and when too much fasciclin II is expressed, excessive fasciculation is produced. Semaphorin I is another cell surface protein necessary for proper axonal fasciculation, but in this case it is produced by non-neuronal cells in the grasshopper limb bud. A stripe of epithelial cells that express semaphorin I is located at a position in the limb at which the pioneer sensory axons that travel toward the central nervous system make a characteristic turn. When semaphorin I is perturbed with a monoclonal antibody, the axons of the two sensory neurons that normally grow together become defasciculated at that position and form anomalous branches. Fasciculation of axons can thus be affected by molecules on the surface of the growing axons or on the surface of the cells that serve as a substratum for them.

reception of a bifunctional molecule is always mediated through Ig superfamily members as a general rule.

In summary, proteins containing Ig domains are present both in vertebrates and invertebrates and have diverse expression patterns and functions: on neurons and glia, from general adhesion molecules to specific axon guidance molecules, from mediating attraction to causing repulsion. Consequently, their mechanism of action involves more than simple adhesion. The Ig domain is a versatile structural motif that is used in different cell–cell or cell–extracellular matrix recognition events, which could trigger different responses in growth cones based on their interaction with different intracellular signaling pathways. The presence in a given protein of a variable number of Ig domains, often in conjunction with other protein domains, points out a remarkable diversity that could plausibly account for a molecular guidance code based only on Ig superfamily molecules. However, this is not the case, and the molecular code for axon guidance is richer in different protein designs than previously thought.

Other general cell surface adhesion molecules can also function in axonal growth. Two other protein families present on the surface of neurons are the cadherins and the integrins (Bixby and Harrys 1991, Reichardt 1992, Takeichi 1991). The cadherins are calcium-dependent adhesion molecules initially identified in vertebrates. They have several calcium binding sites in their extracellular domain, whereas their intracellular domain interacts with the cellular cytoskeleton. Like N-CAM, cadherins have a wide distribution in non-neuronal tissues, but they are also present in neurons. There are several subtypes within the family; two of these, N-cadherin and R-cadherin, promote axonal growth in addition to their prominent role in segregating cell types within tissues. However, the family is rapidly expanding; new cadherins and cadherin-related molecules, called protocadherins, have been identified in both vertebrates and invertebrates (Sano et al. 1993), but their functions have not yet been determined.

The integrins were the first receptors found to mediate axonal growth in neurons cultured over extracellular matrix proteins. The integrins were discovered in vertebrates, but they are present in invertebrates as well. They are composed of two subunits, α and β, each having sev-

On the double personality of some axon guidance molecules

Traffic signs with two different meanings for navigating axons have been found. They are guidance molecules that have opposite effects, depending on the identity of the growrh cone. The receptor on the surface of the growth cone determines whether it approaches or avoids the guidance cue.

Consider the nematode *Caenorhabditis elegans* (Culotti 1994). Two sets of neurons follow circumferential trajectories along the dorsal–ventral axis of the nematode, but in opposite directions. When they reach the ventral or the dorsal midline they change their direction of growth to follow a longitudinal pathway. Three mutations alter the behavior of these circumferential axons. The *unc-5* gene is required for the pioneer growth cones to grow dorsally, the *unc-40* gene for the ones that grow ventrally, and the *unc-6* gene for both kinds of pioneer growth. Both *unc-5* and *unc-40* encode transmembrane proteins; unc-5 is a member of the Ig superfamily, whereas unc-6 is a soluble protein with a portion clearly related to the extracellular matrix protein laminin. An interesting model of guidance has been proposed (Culotti 1994) in which the unc-6 protein is a bifunctional diffusible molecule produced by the ventral midline cells and unc-5 is one of the putative receptors expressed on the surface of neurons that send their axons dorsally. The interaction of unc-6 with unc-5 results in the repulsion of these specific growth cones from the ventral midline (see Figure 4a). Unc-6, however, also appears to act as a chemoattractant for the growth cones that navigate in the opposite direction, and unc-40 is proposed (Culotti 1994) as the potential receptor that mediates this attraction, although the evidence is not as clear.

Unc-6 is part of a family of proteins called netrins, which are found in vertebrates and other invertebrates. Netrin-1 is produced by the cells at the ventral midline of the vertebrate spinal cord and has been demonstrated to attract the growth cones of commissural neurons in culture (Kennedy et al. 1994, Serafini et al. 1994). Consistent with the functions of unc-6, netrin-1 acts as a growth cone repellent as well (Colamarino and Tessier-Lavigne 1995).

The protein J1-160/180 is another protein with a double function. It is an extracellular matrix molecule that has both adhesive and antiadhesive domains. Biochemical and neuronal culture experiments suggest that another protein, F3/F11, an Ig superfamily member, is the receptor mediating the avoidance reaction caused by J1-160/180 (Pesheva et al. 1993). The receptor that mediates positive adhesion has not been found.

Another guidance molecule that seems to lead a double life is connectin, which belongs to a family of proteins with leucine-rich repeat domains, regions rich in the amino acid leucine. Connectin is expressed by a subset of motoneurons and muscles in the fruit fly, and there is evidence that it attracts the growth cones of some motoneurons while inhibiting the approach of others (Nose et al. 1994).

Of all of these bifunctional guidance molecules, only unc-6 and J1-160/180 have candidate receptors. Much more research needs to be done to find the putative attractive receptors for unc-6 and J1-160/180, as well as the receptors for connectin. The bifunctional guidance molecules and their conservation throughout evolution is becoming a fascinating topic for researchers studying axonal pathfinding (Dodd and Schuchardt 1995, Goodman 1994). These molecules are interesting elements of the guidance code, a way of signaling different responses to the same target. They reveal the importance of receptors at the growth cone surface in interpreting a guidance cue and executing a pathfinding decision.

eral different subtypes. Integrin receptors with different ligand specificities can thus be assembled by combining different subtypes of subunits. For example, the receptor $\alpha_3\beta_1$ promotes axonal outgrowth by binding to the extracellular matrix molecule laminin, whereas the integrin $\alpha_1\beta_1$ acts as a collagen receptor (Reichardt 1992).

Neither cadherins nor integrins have been demonstrated to specify the direction of axonal growth. However, most of the research on these two families has been done on neuronal cultures in which the pathway selection by growth cones was not assayed. As with other large families of adhesion molecules, it is still possible that the outgrowth promoting activity of these proteins could be used for guidance at particular places and times during development. For example, integrins expressed on neurons from the retina show an interesting temporal regulation: When they reach the central nervous system some signal from the target region causes a loss in integrin molecules on the surface of these neurons that parallels a loss of the ability to extend axons over laminin (Reichardt 1992). Recently, researchers have generated mice that carry murations in the β_1 and several α subunits of the integrins, as well as in E-cadherin and N-cadherin (Müller and Kypta 1995). Unfortunately, and consistent with the wide distribution of these molecules during development, the main phenotype of the mutants is early embryonic death, thus preventing an analysis of the development of the nervous system. Inactivation of integrins and cadherins in a more restricted way is needed to assay their roles in nervous system development in general and especially in axonal pathfinding.

New families of pathfinding molecules. Fasciclin I was the first cell adhesion molecule described in the invertebrate nervous system. It is expressed by a subset of neurons in grasshoppers and flies and is regionally confined to portions of axons that travel along specific pathways (Figure 3). Like fasciclin II and III, it is a good candidate to be involved in axon guidance, according to evi-

dence from two different approaches. Inactivation of fasciclin I in the grasshopper embryo using the technique called CALI (chromophore-assisted laser inactivation) showed that axons that normally travel together in the limb bud of the embryo become defasciculated when fasciclin I is inactivated, whereas axonal growth and direction are not impaired (Diamond et al. 1993). The second approach has been to generate fasciclin I mutants in the fruit fly (Goodman et al. 1992). As in the case of other cell surface molecules, the absence of fasciclin I in a loss-of-function mutant does not cause any gross abnormality in the nervous system. The same result is obtained when the enzyme Abelson tyrosine kinase, an intracellular signaling molecule that is expressed in axons, is missing. However, when both genes are mutated the embryos show drastic defects in the central nervous system (Elkins et al. 1990). The RP1 neurons, which express both proteins, normally cross the midline and then exit the central nervous system on the contralateral side of the ganglion. In the double mutant, RP1 neurons fail to grow toward the midline. These results favor the hypothesis that axonal pathfinding is the result of a concerted action by several molecular cues, because disruption of the reception of two different signals is required to misroute a particular axon. This apparent redundancy could work to produce a robust pathfinding process that ensures a correctly wired nervous system.

Semaphorin I was discovered in grasshoppers (Kolodkin et al. 1992) as another cell surface molecule restricted to a subset of axons (Figure 3). It was initially thought to represent a unique class of protein, but a subsequent search through many different organisms led to the discovery of a family of related molecules present from viruses to humans (Dodd and Schuchardt 1995, Kolodkin et al. 1993). In addition to its neuronal expression, semaphorin I is found on the surface of epithelial cells localized in stripes in the grasshopper embryonic limb bud (Figure 4b). One of these stripes seems to be an important landmark for the guidance of pioneer sensory neurons. Perturbation of semaphorin I using a monoclonal antibody causes these neurons to defasciculate when they come into contact with the epithelial stripe.

Independent studies searching for molecules that produce growth cone collapse (see Figure 2a) led to the purification and identification of a chicken glycoprotein called collapsin (Luo et al. 1993). Sequence comparisons revealed that collapsin and semaphorin I share a highly similar domain that defines the family. However, semaphorin I transverses the cell membrane, whereas collapsin and other members of the family are secreted proteins with a positively charged region that might bind to negative charges on the plasma membrane. The function of the semaphorin domain is not completely understood, but its presence in a guidance protein in the grasshopper and in a chicken protein with collapsing activity is provocative: Perhaps semaphorin I accomplishes its guidance function through a repulsive mechanism, and perhaps a collapsing factor might function as a guidance molecule.

Several recent studies provide some evidence supporting the latter possibility. An analysis of the growth cone behavior on contact with collapsin-coated beads (Fan and Raper 1995) shows that collapsin can steer growth cones away from the beads (see Figure 2b) without producing full collapse, indicating that collapsin is not only able to inhibit growth but might also reorient growth cones. Moreover, recent work done with human semaphorin III, the equivalent of chicken collapsin, has demonstrated that it specifically repels axons from one group of rat sensory axons in culture without affecting other axons, in a manner consistent with the behavior of those axons in the living embryo (reviewed in Dodd and Schuchardt 1995). These results also suggest that the semaphorin III/collapsin protein can act as an axon guidance molecule. The *Drosophila* semaphorin II, another secreted member of the family, also has an inhibitory role. When semaphorin II is expressed ectopically in two muscles, the growth cone of the motoneuron that innervates them arrests in their vicinity (Matthes et al. 1995). A striking characteristic of the semaphorin family is that it includes both soluble and membrane proteins, suggesting that guidance through repulsion can be achieved either using cell surface cues (contact-mediated inhibition) or chemorepulsion by diffusible repellents.

In our search for cell surface guidance molecules in the grasshopper embryo, we have recently discovered a guidance protein expressed on the surface of a subset of neurons (Figure 3) that is a member of a family until now functionally unrelated to axonal pathfinding (Ganfornina et al. 1995, Sánchez et al. 1995). This protein, lazarillo, is relatively small and has a large carbohydrate moiety. It is attached to the extracellular side of the neuronal plasma membrane through a lipid tail. The amino acid sequence of lazarillo clearly shows that it is a member of the lipocalin family, extracellular soluble proteins that transport small lipids both in invertebrates and vertebrates. Unlike the other lipocalins, lazarillo is clearly involved in the guidance of a particular pair of commissural neurons toward the midline of the embryo (Figure 4a), because perturbation of the protein expressed on these neurons with a monoclonal antibody results in misrouting of their axons.

A new cell surface protein, lazarillo, along with a new protein family, the lipocalins, can thus now be included in the molecular code for pathfinding, and particularly in the code guiding axons toward and away from the midline. This area is the focus of intense research. Several molecules that are produced by midline cells have been found to simultaneously attract commissural growth cones while they repel others (see box).

Continuing the search: more candidate molecules for axon guidance. Two interesting mutations in the fruit fly were found when researchers were looking for alterations in the ganglionic scaffold of axon pathways (Seeger 1994). Mutations in the *commissureless* gene (Figure 4a) result in the absence of nearly all central nervous system commissures.

Axons that normally project across the midline, such as the RP1 or the SP1 neurons, fail to do so, and instead the axons follow a trajectory that is normal but on the wrong side of the embryo. In contrast, the mutation *roundabout* causes axons that do not normally cross the midline to be attracted toward it. These mutations evidently disrupt the attractive and repulsive guidance system that determines which neurons cross the midline and which ones stay on one side of the embryo. The nature of the roundabout protein is not yet known, but the gene encoding the commissureless protein has been cloned. Commissureless is a transmembrane protein found specifically on the surface of the midline cells (Mitchell et al. 1994). The current idea is that in addition to soluble morphogens that would be necessary to attract or repel the growth cones to the midline (see box), direct contact of the growth cones with the surface of midline cells might also be required.

Similarly, several fruit fly mutants (e.g., *beaten path*, *stranded*, and *short stop*) have been identified that are necessary for motoneurons to grow past certain choice points (Seeger 1994). Only the protein encoded by *stranded* has been characterized. It appears to be an intracellular modulator of the cytoskeleton and could have a novel role in regulating growth cone motility. Another mutant, *bendless* (reviewed by Seeger 1994), was identified by using a behavioral assay for a defective escape response. In these mutants, the axons of the giant fiber pathway of the fruit fly fail to make a final lateral bend that normally directs them to the appropriate motoneuron. The inappropriate pathway prevents the formation of the normal synaptic connection. The cloning of the *bendless* gene resulted in an intriguing surprise: The protein is related to ubiquitin-conjugating enzymes (enzymes involved in targeting proteins for degradation, cell cycle control, and modification of protein function), which were previously not known to have any role in growth cone pathfinding.

The nematode *C. elegans*, with its simple nervous system of only 302 adult neurons (compared with the 2000 neurons in a single insect ganglion or the millions in a vertebrate nervous system), is a good model to look for axonal pathfinding mutants. Three mutations, *unc-5*, *unc-6*, and *unc-40*, have received special attention because they affect the guidance of pioneer axons along the dorsal–ventral axis of the embryo. The characterization of these mutations has led to the discovery of soluble (unc-6) and cell surface (unc-5 and unc-40) proteins that are part of a key guidance system that appears to be conserved throughout evolution (see box). However, the role of other elements that are implicated in guidance of axons toward and away from the ventral midline, such as lazarillo, commissureless, or roundabout, needs to be understood. Certainly the midline guidance system is likely to keep drawing the attention of many researchers for years to come.

In addition to the fruit fly and the nematode as model genetic systems, important advances have been made using the zebra fish, with its accessible and transparent embryo. This fish has recently become amenable to genetic technology. Large-scale mutant screens are now being performed (Kuwada 1995), and the search for mutations that affect the axonal projections between the retina and the central nervous system is of special interest. A total of 178 mutations have so far been identified that affect the proper establishment of these connections, an important step in the processing of visual information by the adult brain. Many mutations cause pathfinding errors by the retinal axons on their way to the central nervous system. Others affect how the final contacts between the retinal axons and the neurons in the target area are made. None of these mutations has yet been fully characterized, but they are certainly likely to contribute to the understanding of pathfinding and target recognition in vertebrates.

Fasciclin I and II, semaphorin I, lazarillo, L1, TAG-1, and many others were initially identified using the strategy of generating monoclonal antibodies with suggestive labeling patterns that were subsequently used to isolate these proteins as well as to test their functions. In all searches based on expression patterns, the main criterion used to choose candidates to study has been the spatial or temporal restriction to subsets of cells within the nervous system. The most extreme case of restriction to date is the protein that we have named conulin (Sánchez et al. 1996). It is expressed only on the surface of a subset of neuronal growth cones in the developing grasshopper embryo. Furthermore, these growth cones express conulin only when they are traveling along a specific subset of central nervous system fascicles. The presence of conulin coincides with the times when these growth cones are making specific turns or changing from one bundle to another within the scaffold already set by the pioneer neurons (see Figure 5). Conulin is a cell surface molecule, but its association with the membrane is easily disrupted and the protein is then released into the ex-

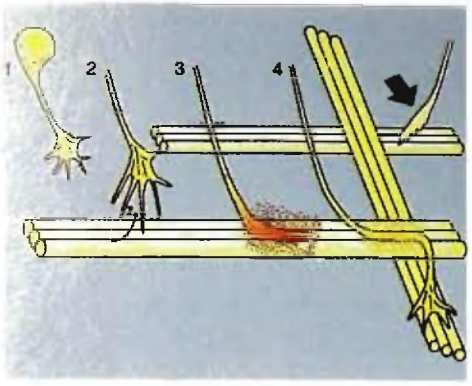

Figure 5. Transient expression of the grasshopper protein conulin on the surface of growth cones is correlated with the recognition of specific axon bundles. Conulin is stored in intracellular vesicles before any protein is localized at the cell surface (step 1). In the next step (2), the vesicles are targeted to the growth cone, probably after the reception of some signal from a particular axon fasicle. Next (step 3), conulin is localized at the surface and is also released from the growth cone. Finally (step 4), conulin is turned off when the growth cone changes from one fascicle to another. Other growth cones that follow different axon bundles (green arrow) never express conulin. The narrow temporal and spatial window of conulin expression makes it an interesting new candidate to regulate recognition processes during axonal pathfinding.

tracellular space. We do not yet know whether conulin is released to perform an active function by modifying the environment surrounding the growth cones, or whether the release is part of a dynamic cycle to restrict its presence on the growth cone surface to a limited time window. All of these aspects of the expression pattern and turnover of conulin certainly suggest a role for conulin in the specific recognition events that must occur between the subset of growth cones that express it and the axonal fascicles they navigate along.

Conclusions

These are certainly exciting days for developmental neurobiologists as the understanding of the journey an axon takes to find its target is finally unfolding. Interesting insights are coming from both cellular and molecular research. A multitude of new techniques is successfully being used to describe growth cone activities, not only in culture systems but also in intact embryos. In addition, many new guidance molecules are being discovered, each one adding a piece to a puzzle in which many protein designs are involved. Because many molecular mechanisms appear to be conserved throughout evolution, the molecular guidance code for neuronal pathfinding is also expected to be generally conserved. An important element in the information processing required for all pathfinding decisions has proven to be the surface of growth cones and of the cells they contact during their navigation. Consequently, the cell surface has awakened much research interest. We believe that deciphering this molecular guidance code will be a key point in understanding the development of the nervous system and hence its astonishing functional capabilities.

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